Natural Fluorescence of Red and White Wheat Kernels

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ABSTRACT

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Red and white wheats must be segregated for marketing purposes because they have different end uses. Identification of wheat color is not straightforward, and currently there is interest in characterizing red and white wheats using spectroscopic methods and chemical tests. The kernels of both red and white wheats exhibit natural fluorescence that can be readily viewed under UV light, although it is not possible to differentiate the fluorescence spectra of red and white wheats by visual inspection only. Fluorescence emission spectra in the wavelength range of 370–670 nm for 91 wheat samples consisting of 48 red (from 30 cultivars) and 43 white (from 18 cultivars) were analyzed by partial least squares (PLS) and neural networks analyses (NNA). Samples included cultivars that were difficult to classify visually as well as wheat harvested after rainfall. Classification accuracies were ≈85% for calibration and ≈72% for the validation samples by both analyses. A plot of β-coefficient vs. wavelength in PLS analysis indicated that fluorescence of red wheat cultivars was greater than that for white wheat cultivars at 425 (±20) nm wavelength. Fluorescence of white wheat cultivars was greater than that for red cultivars at 587 (±35) nm. Fluorescence emission at ≈450 nm from wheat samples increased in intensity after treatment with NaOH. The increase was greater for red than for white wheat. Wheat harvested after rainfall also exhibited a slight increase in fluorescence.

Red and white wheats must be segregated for marketing purposes. Mixtures are usually discounted and some of the wheats are intended for different end uses. Both hard red and hard white wheat cultivars may be used for bread, but some white wheats are also used for Asian noodles. Identification of wheat color is not straightforward, and grain handlers and farmers are interested in characterizing red and white wheat by using rapid spectroscopic or chemical tests (Dowell 1997, 1998; Ram et al 2002a).

Intrinsic fluorescence has been suggested for quantification of botanical components in wheat (Jensen et al 1982; Jensen and Martens 1983; Symons and Dexter 1993). Fluorescence microscopic studies indicated a correlation of the autofluorescence with wheat hardness (Irving et al 1989), but these studies did not include samples of hard white wheat. Intense blue fluorescence of the aleurone cell walls of wheat is due to high concentrations of ferulic acid (Fulcher et al 1972; Fulcher and Wong 1980). Other fluorescence and UV absorption studies also indicate the presence of ferulic acid in cell wall fluorescence (Pussayanawin et al 1988; Akin 1995; Collins and D'Attilio 1996). McKeehen et al (1999) identified a potential association between phenolic acid concentrations and *Fusarium* resistance. Several other compounds in wheat were identified in these studies, but none were described as contributing to autofluorescence.

Three emission bands in fluorescence from cereal flours have been reported (Zandomeneghi 1999). The most intense at ≈330 nm (excitation 280 nm) was attributed to amino acids. The band at 430 nm (excitation 330 nm) was ascribed to tocopherol and related compounds, and the band at 540 nm (excitation 445 nm) was assigned to a xanthophyll. These results have not been corroborated. Flavonoids have been reported in wheat germ milling fractions (Barnes et al 1987; Barnes and Tester 1987).

The fluorescence of whole wheat kernels had not been examined previously. We were interested in examining differences in the surface emissions from red and white wheat kernels. Some other grains are segregated by fluorescence in breeding programs. For example, white and yellow oats are viewed in UV boxes by oat breeders, and annual and perennial rye grasses are segregated on the basis of fluorescence of the primary root during the early stages of germination. Also, neural network analysis (NNA) has been used recently to classify genetics of barleys based on phenolic finger prints (Gorodkin et al 2001).

MATERIALS AND METHODS

Wheat samples used in this study are listed in Table I (red wheat cultivars) and Table II (white wheat cultivars). Most of these samples were used in previous studies (Dowell 1997, 1998; Ram et al 2002a); the other wheat cultivars were harvested in 2000-2002 and were obtained from Joe Martin, Kansas Agricultural Experimental Station, Hays, KS. The color classes of wheat samples that were not obviously red or white were determined using the NaOH soak test (Ram et al 2002a). The sample set contained 11 samples that were difficult to color classify. Samples were stored at 4°C to avoid mold and insect infestation.

Fluorescence

Wheat kernels were viewed under long-wavelength UV light (360–400 nm), referred to as blacklight, in a Spectroline CX-20 UV cabinet. For magnified viewing, a laboratory microscope with up to 50× magnification was used with a blacklight (model B-100A, Ultraviolet Products, San Gabriel, CA) excitation.

Fluorescence emission spectra were obtained from bulk samples using a FluoroMax-2 spectrofluorometer (Jobin Yvon Spex, Edison, NJ), with 1-nm resolution and 1.0-sec integration time. The instrument had one monochromator for excitation and one for emission. Instrument control and data acquisition were computer-controlled and spectra were saved in Grams/32 (Thermo Galactic, Salem, NH). Fluorescence spectra of whole wheat kernels were obtained using a 10-mm path-length quartz spectrophotometric cuvette that held ≈30 kernels. No special adaptation to increase the signal from solids such as described by Zandomeneghi (1999) was used. Emission spectra (370–670 nm) were obtained with 350 and 300 nm excitation. Broken kernels, straw, and chaff were removed, and only whole kernels were used. Most cultivars were scanned once, but more than one sample was used for a few cultivars, as noted in Tables I and II.

PLS Analysis

Fluorescence data of red and white wheat were analyzed using partial least squares (PLS) analysis (Martens and Naes 1989). All

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TABLE I
Partial Least Squares (PLS)[a] and Neural Network Analyses (NNA)[b] of 370–670 nm Fluorescence Emission Data for Red Wheat Cultivars[c]

Red Wheat Cultivar	PLS Prediction	NNA Output PE1 Value	Red Wheat Cultivar	PLS Prediction	NNA Output PE1 Value
Calibration set[d]			Ogallala	1.20	0.89
2137 (1)[f]	1.35	1.32	Prowers	1.25	0.74
2137 (2)	0.81	0.78	Scout66	1.12	0.88
2137 (3)	1.25	0.83	Scout66-1	1.19	0.88
2163	1.24	0.90	Tam107	1.86	0.38
2172	1.25	0.88	Tam110	1.51	0.62
2174(1)	1.15	0.83	Vista	1.19	0.85
2174 (2)	1.19	1.00	Wesley	1.40	0.56
2174 (3)	1.57	0.90	Yuma	1.17	0.95
Akron (1)	1.16	0.99	**Validation set[e]**		
Akron (2)	1.16	0.84	2174-1	1.33	0.65
Arapahoe-1 (1)[g]	1.05	0.93	2174-3	1.25	0.78
Arapahoe-1(2)	1.07	1.09	Arapahoe-1 (3)	1.14	0.90
Big Dawg-1 (1)	1.29	0.62	Arapahoe-3	1.17	0.87
Big Dawg-1 (2)	1.30	0.72	Big Dawg-3	1.13	0.92
HRS bleached[h,i]	1.10	0.88	FGIS-1	1.67	0.29
Ike-1 (1)	1.18	0.87	FGIS-2	2.41	-0.22
Ike-1 (2)	1.00	1.28	KS75216-3	1.44	0.41
Jagger (1)[i]	1.27	0.93	Ike-3	–1.13	0.15
Jagger (2)[i]	1.06	0.92	Karl	1.50	1.33
Jagger (3)[i]	1.38	0.59	KCI R/W	1.73	0.44
Karl92[i]	1.30	0.77	Neeley	1.35	0.52
KCI R/W[i]	1.23	0.77	Pronghorn	0.73	1.04
KS75216-1	1.39	0.53	Rampart	1.34	0.65
KS84HW196	1.36	0.80	Scout66-3	1.20	0.75

[a] PLS prediction value <1.41 indicates red wheat.
[b] NNA output PE value 1 > 0.5 indicates red wheat.
[c] All cultivars are from Kansas 1999-2002. Analysis of 350 nm excitation data.
[d] Prediction values for the calibration set are those of cross-validation.
[e] Independent values for the validation set are those predicted by the calibration model.
[f] Numbers in parentheses indicate replicate spectrum number using this cultivar.
[g] Cultivar name with -1 suffix harvested before rainfall; with -3 suffix harvested after rainfall.
[h] Cultivar name unknown, not the name of the cultivar.
[i] Contrasting color class appearance difficult to color classify.

TABLE II
Partial Least Squares (PLS)[a] and Neural Network Analyses (NNA)[b] of 370–670 nm Fluorescence Emission Data for White Wheat Cultivars[c]

White Wheat Cultivar	PLS Prediction	NNA Output PE2	White Wheat Cultivar	PLS Prediction	NNA Output PE2
Calibration Set[d]			**Validation Set[e]**		
Argent[f]	2.41	1.29	Arlin (1)	1.83	0.93
Arlin-1[g]	1.60	0.89	Arlin (2)	1.74	0.83
Arlin-3	1.15	1.01	Betty (1)	1.50	0.61
Betty (1)[h]	1.80	0.91	Betty (2)	1.60	0.58
Betty (2)	1.36	0.44	Heyne (1)	1.17	0.32
Heyne-1 (1)	1.59	0.88	Heyne (2)	1.32	0.10
Heyne-1 (2)	1.34	0.48	Heyne-3	1.19	0.15
Heyne-1 (3)	2.46	1.33	KS84HW196	1.50	0.55
Heyne-1 (4)	1.37	0.51	KS96HW94	1.74	0.74
Klasic	2.12	1.22	KS96HW10	1.38	0.39
KS95H167	1.27	0.21	NuPlains-3	1.69	0.72
KS96HW115	1.86	1.12	Oro Blanco-1	1.48	0.53
Lakin	1.61	0.76	Oro Blanco-3	1.24	0.19
NuPlains (1)	1.75	1.07	Rio Blanco (1)	1.91	0.97
NuPlains (2)	2.24	1.17	Rio Blanco (2)	1.71	0.80
Oro Blanco (1)	1.43	0.54	Trego (1)	1.62	0.67
Oro Blanco (2)	1.46	0.42	Trego (2)	2.07	1.27
Oro Blanco-1	1.34	0.59	Trego (3)	2.45	1.17
White Eagle-1 (1)	1.62	0.83	White Eagle-1 (3)	1.67	0.76
White Eagle-1 (2)	1.52	0.43	White Eagle-3	1.68	0.72
White Chief-1 (1)	1.38	1.06			
White Chief-1 (2)	1.84	0.36			
White Chief-3	1.41	0.75			

[a] PLS prediction value >1.41 indicates white wheat.
[b] NNA output PE value 2 > 0.5 indicates white wheat.
[c] All cultivars are from Kansas 1999-2002. Analysis of 350 nm excitation data.
[d] Prediction values for the calibration set are those of cross-validation.
[e] Independent values for the validation set are those predicted by the calibration model.
[f] Contrasting color class appearance difficult to color classify.
[g] Cultivar name with -1 suffix harvested before rainfall; with -3 suffix harvested after rainfall.
[h] Numbers in parentheses indicate replicate spectrum number using this cultivar.

data were mean-centered. Spectral data were analyzed by the Grams/32 PLS model using the emission data in the 370–670 nm range (obtained with excitation at 350 nm) at 1-nm intervals (*x*-data) and appropriate constituent values (*y*-data) using a full cross-validation scheme. The assigned constituent values for red and white wheats were 1 and 2, respectively. The number of factors reported was obtained from PRESS (predicted residual error sum of squares) vs. the number of PLS factors plot corresponding to the lowest PRESS value obtainable with the least number of factors. This number of factors, which was close to the software recommended value, was evaluated as acceptable from actual vs. predicted and β-coefficient vs. wavelength plots.

We divided our sample set into a calibration and a test (or validation) set of samples. The calibration set consisted of 33 red wheat and 23 white wheat samples in a total of 56 samples, and the validation set consisted of 15 red and 20 white wheat samples in a total of 35 samples.

Neural Networks Analysis

A software package (Professional II/Plus, Neuralware, Pittsburgh, PA) was used for data analysis on a back-propagation network (Hecht-Nielsen 1989; Wang et al 2002). Neural network models without any hidden layers were developed. Fluorescence emission data (370–670 nm obtained with 350 nm excitation) at 2-nm intervals were input as 151 processing elements (PE values or variables). There were two output values corresponding to red and white wheat and these were input as (1,0) and (0,1), respectively. The optimum neural network parameters were learning cycle of 10,000 (179 cycles/spectrum), learning rate of 0.7, and momentum of 0.5. Optimization procedures for these factors have been described previously (Wang et al 2002).

RESULTS AND DISCUSSION

Fluorescence

All cultivars of red and white wheats listed in Tables I and II exhibited natural fluorescence when viewed under UV light in a viewing cabinet. Compared with red wheat, white wheat appeared to have more intensity and a whiter fluorescence, which may be due to less quenching in the white wheat. This natural fluorescence was not uniform on the surfaces of the kernels. Under magnification, many kernels exhibited distinct patches of blue fluorescence, which may be due to portions of the aleurone layer being visible through the pericarp. Fluorescence variations among wheat kernels may also be due to morphological variation in the pericarp and nucellar organization, both of which have their own fluorescence characteristics (Fulcher et al 1972; Fulcher and Wong 1980; Irving et al 1989; McKeehan et al 1999).

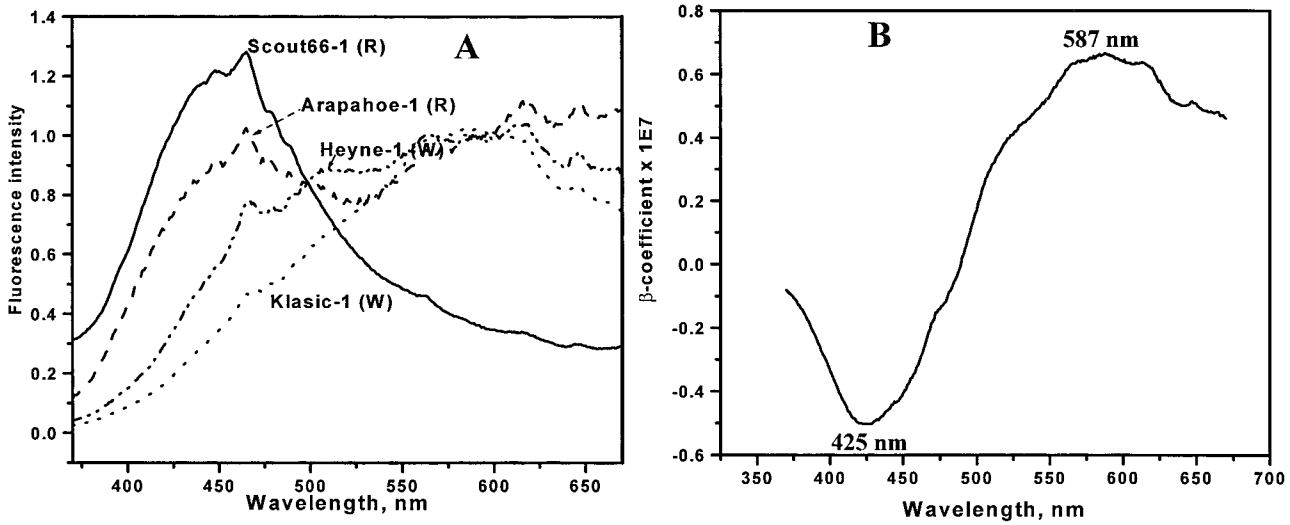

Fig. 1. A, Fluorescence emission spectra (obtained with 350 nm excitation) of kernels of red (R) and white (W) wheat cultivars: Scout66-1 (R), Arapahoe-1 (R), Klasic-1 (W), and Heyne-1 (W). **B,** Plot of β-coefficient vs. wavelength for red vs. white wheat classification by PLS of fluorescence spectra.

TABLE III
Classification of Red and White Wheat Cultivars by Partial Least Squares (PLS) and Neural Network Analyses (NNA) of 370–670 nm Fluorescence Emission Spectra with Excitation at 350 nm

PLS		NNA	
Wavelength range	370–670 nm	Wavelength range	370–670 nm
Number of points	301	Number of points	151
Number of factors used	2	Momentum, learn rate	0.5, 0.7
Data preprocessing	None	Learn counts/spectra	179
Number of calibration samples	56 (33 red, 23 white)	Data preprocessing	None
Number (%) of red wheat spectra correct in calibration set (predicted value < 1.41)	30 (91%)	Number of calibration samples	56 (33 red, 23 white)
		Number (%) of red wheat spectra correct (output PE value 1 > 0.5)	32 (97%)
Number (%) of white wheat spectra correct in calibration set (predicted value > 1.41)	16 (70%)	Number (%) of white wheat spectra correct (output PE value 2 > 0.5)	17 (74%)
Total calibration samples correct (PLS)	46 (82%)	Total calibration samples correct (NNA)	49 (88%)
Number of validation samples	35 (15 red, 20 white)	Number of validation samples	35 (15 red, 20 white)
Number (%) of red wheat spectra correct in validation set	10 (67%)	Number (%) of red wheat spectra correct (output PE value1 > 0.5)	11 (73%)
Number of white wheat spectra correct in validation set	15 (75%)	Number (%) of white wheat spectra correct (output PE value 2 > 0.5)	15 (75%)
Total validation samples correct (PLS)	25 (71%)	Total validation samples correct (NNA)	26 (74%)

In a cross-section of a wheat kernel, a blue fluorescence similar to that observed on the surface was concentrated in the nucellus and aleurone layers which are both very fluorescent. A weak blue fluorescence was also dispersed in the endosperm, probably because the endosperm cell walls also contain low concentrations of ferulic acid and ferulates. It is possible that the fluorescence observed in this study may not be merely from the surface of the kernel because the incident UV light may penetrate into the kernel and cause fluorescence from inside the kernel to emanate out. Overall, the observed fluorescence was likely a function of 1) the types and concentrations of each fluorescent compound and the morphology of the several cell layers that comprise the outer structures (aleurone, nucellus, testa/seed coat, and the several pericarp layers) of each kernel, and 2) the physical characteristics of each kernel such as size, shape, and surface texture. All of these parameters very likely influenced the reflectance profile of each fluorescence spectrum, at least to a level of affecting the values utilized by the PLS and NNA procedures.

Whole wheat kernels gave only weak fluorescence spectra, and the intensity and the wave characteristics were possibly affected by the packing and orientation of the kernels in the cuvette. Due to limited experimental resources, only one spectrum was recorded for each sample, although we made repeat measurements with repacks for a few samples (Tables I and II). Fluorescence emission with 350 nm excitation was more intense than with 300 nm excitation for all samples. Fluorescence emission data obtained with 350 nm excitation was used in PLS and NNA. Typical emission spectra from red and white wheat samples are illustrated in Fig. 1A. Red wheats exhibit stronger fluorescence intensity in the 370–470 nm region than in the 470–680 nm region, while white wheats fluoresce more strongly between 470–680 nm than at 370–470 nm. However, it was not always possible to discriminate between red and white wheat by visual examination of their fluorescence spectra alone. The number of spectra obtained from different samples of the same cultivar is indicated in Tables I and II.

PLS Analysis

Fluorescent spectra of wheat samples were analyzed by PLS with and without normalization of the fluorescent intensity. Because of the large variation in the intensity of the fluorescence spectra, we thought it might be helpful to normalize the spectra and then analyze the modified data set by PLS or NNA.

When all samples were included, normalization of the spectra helped convergence in PRESS values with increasing number of factors. Classification accuracies of PLS cross-validation results for the normalized set were 88% of all 91 samples (88% of 48 red wheat samples and 88% of the 43 white wheat samples).

Initial attempts to develop a calibration model failed until several outliers were removed. Outlier samples usually arise from some incorrect measurement, whether it is in the concentration data (i.e., errors in the primary calibration technique, transcription errors) or in the spectral data (i.e., spectrometer error, sample handling procedures, environmental control such as temperature, humidity, etc.). In the simplistic approach we used for measuring fluorescence of wheat kernels, orientation and packing of kernels in cuvette could lead to outliers. Including outlier samples also introduces a bias to the model leading to incorrect predictions. Also, a calibration with a high value of N/V (≈10), where N is the number of samples and V is the number of variables, can reliably predict unknowns.

Predicted constituent values from PLS are cross-validation values for the calibration set. The PLS calibration model was used to predict the constituent values of the validation set that were not part of the calibration set. These values for red and white wheat cultivars are given in Tables I and II. For classification, predicted constituent values had to be above or below a set threshold value. Predicted values >1.41 were considered spectra of white wheat, and those <1.41 were considered spectra of red wheat. The value 1.41 is a correction applied to the value of 1.5 for equal number of red and white samples. PLS results are summarized in Table III.

Classification accuracies were 91% for red and 70% for white wheat cultivars for the calibration set and 67% for red and 75% for white wheat in the validation set. Considering that the calibration was done with limited samples and many of the samples in the test set were outliers from preliminary calibrations, the slightly lower prediction accuracy for the test set is not surprising.

A plot of β-coefficients of the PLS analysis vs. fluorescence wavelength is shown in Fig. 1B. Wavelengths corresponding to the maximum (587 ± 35 nm) and minimum (425 ± 20 nm) are important for classification of spectra. A comparison of Fig. 1 A and B, shows how the β-coefficients in PLS analysis are related to the actual emission spectra of red and white wheat. Previously, we have made a similar comparison of a plot of the β-coefficients in PLS analysis with the NIR spectrum of a coating in wheat (Fig. 3 in Ram et al 2002b). At ≈587 nm, white wheats exhibited more fluorescence than red wheats, and at ≈425 nm, red wheats exhibited more fluorescence than white wheats.

Neural Network Analysis

NNA was conducted using fluorescence data taken 2 nm apart, that is, 151 input PE values (N/V = 0.59). NNA returned two output PE values for each sample spectrum. The sample set used in PLS calibration set was used to train the network. The trained network was used to test the unknowns in the same test set that was used in PLS validation. The output PE values for the learning set (same as the PLS calibration set) and the test set (same as that used in PLS) are given in Tables I and II for the red and white wheat cultivars. The value of the first PE was greater than that for the second for red wheat cultivars and vice versa for the white wheats. A summary of the results of NNA is presented in Table III. NNA classification accuracies were 97% for red and 74% for white wheat cultivars for the calibration set and 73% for red and 75% for white wheat in the validation set. As we stated earlier for PLS analysis, considering that the calibration was done with limited samples and many of the samples in the test set were outliers from preliminary calibrations, the slightly lower prediction accuracy for the test set is not surprising.

Tables I and II show that NNA prediction is very similar to PLS prediction for every sample. PLS analysis is a linear regression model and NNA is for linear and curvilinear models; similarities in prediction values by the two methods for each and every sample of red and white cultivars could be noted.

Analysis of spectra of 89 samples by NNA with 31 input PE values (thus improving the samples/variables ratio, which generally improves the reliability of the prediction values, to 2.87) using fluorescence data 10 nm apart, yielded nearly the same results as with 151 PE values. Results nearly identical to that given in Table III for 151 PE values or variables (data every 2 nm apart) were obtained. Fluorescence emission bands were broad, and including data points 10-nm apart, was sufficient for good classification.

Factors Affecting Analysis of Fluorescence Spectra

A number of factors affected the analysis of spectra. The emission intensity was variable. The variations in intensities were probably due to how the kernels were packed and oriented in the cuvette. Rainfall before harvest also affected the fluorescence of the samples. Kernel morphology, grain dust, broken kernels, chaff, and straw contamination contributed to the emission spectra. Also, if a wheat sample contained a low percentage of kernels of contrasting color, it was possible that the emission spectrum could be modified by the minor component. Most white wheat kernels typically had 2–5% red wheat mixed in them and, occasionally, there was white wheat mixed in red wheat as well. Considering these factors, the correlation of the wheat color class with the fluorescence emission spectra is good. Other factors that affected the classification here were 1) the sample set included a

large number of samples that were difficult to classify visually and rain-bleached samples, and 2) not having multiple spectra for each of the samples (because of the variability in the spectra).

Characterization of fluorescent compounds extracted from the surfaces (bran layers) of red and white wheat is currently being studied. Some *p*-hydroxycinnamic acids such as ferulic, coumaric, and caffeic acids appear to be responsible for fluorescence at ≈425 nm. Fluorescence in the range 550–610 nm could possibly arise from flavonoids like apigenin (≈540 nm green), quercetin (≈580 nm yellow), and luteolin (≈600 nm, red), or from carotenoids such as xanthophylls and lutein (Zandomeneghi 1999). It appears there are slight differences in both categories of compounds in red and white wheat.

Wheat samples exhibited greater fluorescence after the NaOH test (Fig. 2). Red wheat samples had a larger increase in fluorescence intensity than white wheat samples. In red wheat samples, fluorescence at higher wavelengths appeared washed out. Both red and white wheat samples appeared coated with ferulic acid (and perhaps other compounds not contributing to the net fluorescence) after the NaOH test, based on the appearance under the blacklight and the fluorescence spectrum ($\lambda_{max} \approx 450$ nm).

Effect of Rainfall Before Harvest on Fluorescence

The effect of rainfall on the fluorescence spectra of wheat harvested after rainfall was variable. The effect was similar to that described for the NaOH test above but much weaker. Generally, fluorescence increased slightly more, particularly with emission at ≈450 nm, for red wheat samples than for white wheat samples.

CONCLUSIONS

All cultivars of red and white wheats that were tested exhibited natural fluorescence when viewed under a UV light in a viewing cabinet; fluorescence was not uniform on the surfaces of the kernels. This fluorescence may be due to components from within the whole kernel and not merely from the epidermal layers, and it may be influenced by physical characteristics of the kernels. Although weak in intensity, the fluorescence spectra of bulk samples of red and white wheats were different. Ninety-two fluorescence spectra of red and white wheat cultivars were classified by PLS and NNA analyses of their natural fluorescence spectra. Further studies may involve use of spectrofluorometers with fiber optic cables and plate readers to determine whether the fluorescence could classify single kernels of wheat according to their color class.

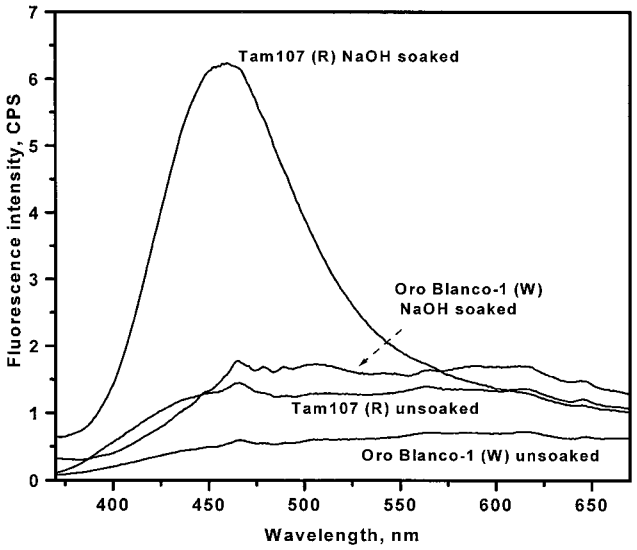

Fig. 2. Fluorescence emission spectra (obtained with 350 nm excitation) of red (R) and white (W) wheat before and after treatment with NaOH. Representative samples are Tam107 (R) and Oro Blanco (W).

This research shows there is some potential for fluorescence to be practical for distinguishing red and white wheat. Spectrophotometers are not expensive, chemicals are not needed, and there is no sample preparation in this method. However, one might need to collect several spectra of the same sample to obtain correct classification.

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